

**ANALYSIS OF DIOXYGENASE GENE IN MARINE BACTERIA
INVOLVED IN POLYCYCLIC AROMATIC HYDROCARBON
DEGRADATION**

**DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR
THE DEGREE OF
MASTER OF SCIENCE IN LIFE SCIENCE**



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DECLARATION

I hereby declare that the major thesis entitled “**Analysis of dioxygenase gene in Marine bacteria involved in Polycyclic aromatic hydrocarbon Degradation**” submitted to Department of Life Science, National institute of technology, Rourkela in the partial fulfillment of the requirement for the degree of Master of Science(Life Science) is an authentic record of the work carried out by me and no part of this thesis has been presented for the award of any degree, diploma, fellowship or any other similar title.

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III. ABSTRACT

The degradation of polycyclic aromatic hydrocarbons (PAHs) by bacteria has been broadly studied. While many pure culture have been isolated and characterize for their ability to grow on PAHs, limited in turn is available on the diversity of microbes involved in PAH degradation in the environment. Pure bacterial cultures were isolated from a highly enriched consortium for biodegradation studies. Bacterial isolates capable of degrading Pyrene and Anthracene were isolated from Paradeep estuary (Odisha) water sample by selective enrichment. Five isolates of pyrene and anthracene degrading pure cultures were obtained, designated as MP-1, MP-4, MP-9, MP-14,MP-18. Isolates were characterized by gram staining, citrate utilization, sugar fermentation, swimming and swarming motility and antibiotic sensitivity test. In 7 days MP-1,MP-4,MP-9,MP-14,MP-18 found to degrade 58.4%, 42.1%, 29.1%, 31.7%, 31.8% at 100 mg/l concentration of Pyrene and 56.3%, 46%, 44.6%, 30.4%, 48.2% at 100mg/l concentration of Anthracene. Polymerase chain reaction (PCR) amplification with PAH-specific primers revealed the presence of a dioxygenase gene in MP-4. The presence of dioxygenase gene may provide opportunities to explore how bacteria develop the abilities to degrade high-molecular-weight polycyclic aromatic hydrocarbons. The ability to profile not only the bacterial community but also the dioxygenases which they encode provides a powerful tool for both assessing bioremediation potential in the environment and for the discovery of novel dioxygenase genes.

Keywords: Biodegradation, Pyrene, Anthracene, marine bacteria, Dioxygenase gene.

1. INTRODUCTION

Biodegradation is a viable bioremediation technology for organic pollutants. It has identified that microorganisms degrade ecological pollutants in various matrices and environments. Bioremediation utilizes the metabolic adaptability of microorganisms to degrade hazardous pollutants. A purpose of bioremediation is to transform organic pollutants into harmless metabolites or mineralize the pollutants into carbon dioxide and water (Alexander, 1985). A feasible remedial technology requires microorganisms being capable of quick adaptation to and efficient uses of pollutants of interest in a particular case in a logical period of time. Many factors influence microorganisms to use pollutants as substrates or to metabolize them. Therefore, understanding catabolic pathways and mechanisms and responsible enzymes is an effective means to define important factors for efficient cleanup of pollutants. Research has been conducted to recognize bioremediation for environmental pollutants such as aromatic compounds that are among the most prevalent and persistent environmental pollutants. Biodegradation is a very wide field and involves uses of a wide range of microorganisms to break chemical bonds. It has been well reviewed (Klein, 2000), however, it is a very active field and new data are rapidly contributed to the literature.

PAHs (Polycyclic aromatic hydrocarbon) are aromatic hydrocarbons with two or more fused benzene rings. They are formed during the thermal breakdown of organic molecules and their succeeding recombination. Partial combustion at high temperature (500–800 °C) or subjection of organic material at low temperature (100–300 °C) for long periods lead to PAH production. They occur as colorless, whitish, yellow solids with low solubility in water, high melting, high boiling points and low vapour pressure. PAHs are environmental pollutants that are found in several polluted soils as a result of natural or industrial activities, as well as those of creosote wood-treatment facilities (Mueller et al., 1989). PAHs have involved considerable attention as a result of their potential toxicity for higher organisms and resistance to microbial degradation (Kanaly and Harayama, 2000). A wide range of micro-organisms have been discovered that are able to degrade highly stable, lethal organic compounds like polycyclic and aliphatic hydrocarbons (Kanaly and Harayama, 2000; Habe and Omori, 2003; Van Hamme et al., 2003). Among these micro-organisms, numerous *Arthrobacter* species are able to degrade PAHs (Grifoll et al., 1992; Seo et al., 2006). Due to their occurrence, recalcitrance, bioaccumulation potential and carcinogenic activity; the PAHs have gathered significant environmental concern.

Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation. Microbial degradation is the major degradation process (Bumpus, 1989; Yuan et al., 2001). PAHs degradation depends on the environmental conditions, number and type of the microorganisms, nature and chemical structure of the chemical compound being degraded. They are biodegraded into less complex metabolites, and through mineralization into inorganic minerals, H₂O, CO₂ (aerobic) or CH₄ (anaerobic) and rate of biodegradation depends on pH, temperature, microbial population, oxygen, accessibility of nutrients, chemical structure of the compound, cellular transportation properties, and chemical partition in growth medium (Singh and ward, 2004).

Aerobic degradation of lower molecular weight PAHs such as naphthalene (NP) by cultured microorganisms has been studied extensively (Cerniglia, 1992). Bacterial degradation of PAHs under aerobic conditions begins with the addition of both molecules of molecular oxygen to the aromatic ring by a dioxygenase. Aromatic ring dioxygenases are multicomponent enzymes which consist of an electron transport chain containing a ferredoxin and a reductase and a terminal dioxygenase (Gibson and Parales, 2000). The dioxygenase is composed of 2 subunits. The α subunit is the catalytic component and contains 2 conserved regions: the [Fe₂-S₂] Rieske center and the mononuclear iron containing catalytic domain. The Rieske cluster accept electrons from the ferredoxin and passes them on to the mononuclear iron for catalysis (Parales, 2003; Ferraro et al., 2005). The majority of information on PAHs degradation pathways has come from studies on gram negative bacteria, particularly the *Pseudomonads* (Simon et al., 1993; Bosch et al., 1999). Microbial degradation of PAHs is considered to be the major decomposition process for these contaminants in nature and represents a potential solution to the environmental problems posed by these chemicals. In recent years, PAHs biodegradation studies have received much attention and many related reports have appeared PAH dioxygenase and catechol 2,3-oxygenase are identified as two key PAH degrading- related enzymes (Resnick et al., 1996; Meyer et al., 1999). The dynamic fate of intermediate metabolites, such as 1-hydroxy-2-naphthoic acid, during PAH biodegradation are very scarce (Grifoll et al., 1995).

As a result, diverse dioxygenase genes for catabolism of aromatic hydrocarbons from one- to multi-ring aromatic compounds have been identified in microorganisms. Mainly, dioxygenase genes for dissimilation of aniline, carbazole, dioxin, phenanthrene, or phthalate have been identified and characterised in a few microorganisms (Batie et al., 1987; Gurbiel et al.,

1989: Happe et al., 1993: Fujii et al., 1997: Fukmori & Sain, 1997: Shepherd et al., 1998: Laurie & Lloyd, 1999: Saito et al., 2000: Eaton, 2001: Nojiri et al., 2001: Moser et al., 2001). A few recent studies on genetic diversity have been mainly conducted with microbial populations or many bacteria. Basically it focuses on analyzing diversity of dioxygenase genes of a bacterium, *Pseudomonas rhodesiae* KK1 adequate of utilizing a broad range of monoaromatic compounds including polycyclic and heterocyclic aromatic hydrocarbons such as carbazole and naphthalene previously mentioned (Kahng, 2002). There are several mechanisms or combination by which microbial communities can adapt to the presence of PAHs in the surroundings. Firstly, there can be an increase in population size of those organisms that tolerate or even degrade the compound by induction of appropriate gene. Secondly, the cells can adapt through mutation of various type such as single nucleotide changes or DNA rearrangements that results in resistance to or degradation of the compound.

Various types of heterocycles comprising oxygen, nitrogen, and sulfur are found in the environment, developing from anthropogenic or instinctive sources. Dibenzofurans, dibenzodioxins, and dibenzothiophene are within the most important environmental pollutants and well reviewed by (Klein, 2000: Nojiri and omori, 2002). Therefore, only aerobic bacterial degradation of non-halogenated heterocyclic aromatics is briefly discussed in this to make a general relation with PAH degradation. Many bacterial species have been described to decompose dibenzofurans and carbazole, a morphological analogue with nitrogen instead of oxygen (Nojiri et al., 2002: Seo et al., 2006) although some white rot fungi are well known to decompose halogenated dibenzofurans isolated a stable carbazole-degrading microbial consortium consisting of *Chryseobacterium* sp. NCY and *Achromobacter* sp. NCW. As the degradation of unsuitable PAHs, bacterial catabolism of dibenzofurans starts at insertion of two oxygen atoms catalyzed by dioxygenases. Although numerous bacterial PAH dioxygenases are evolutionarily related to phenyl propionate dioxygenase, striking diversities have been reported in catalytic activity, mechanism, regulations, and substrates of these crucial enzymes. In short, initial reactions of dibenzofuran and carbazole can be classified into angular and lateral dioxygenation, which may be catalysed by different enzymes. These enzymes were found in Gram positive and negative bacteria (Habe et al., 2003).

However, recent studies with cloned dioxygenase from *Norcardioides aromaticivorans* IC177 revealed that some dioxygenase can catalyze both reactions. It is well known that PAH

dioxygenases can catalyze several reactions, including reduction, mono- and di-oxygenation (Resnick et al., 1996). In addition to the multiple reactions with specific dioxygenases, current genomic or proteomic search with several PAH degrading bacteria (e.g., *Burkholderia* spp. and *Mycobacterium* spp.) exposed that multiple dioxygenases, probably playing dissimilar roles in PAH degradation, exist in a single bacterium (Resnick et al., 1996; Liang, 2006). Multiple catabolic pathways can produce various metabolites with structural diversities, which probably are metabolised by different types of enzymes. Dioxygenases involving in dibenzo-*p*-dioxins degradation catalyze predominantly an angular dioxygenation to produce tri-hydroxy diphenyl ethers. PCDDs are well known pollutants with extravagant toxicity, particularly tetra to hexachloro analogues. Some bacterial species can catabolise upto hexachlorodibenzo-*p*-dioxins (Habe et al., 2002).

Alkyl- and nitro-PAHs are among common substituted PAHs and have significant toxicities. Commutation on aromatic rings produces several problems in their degradation. The power of PAH dioxygenase to remove the substitutions is currently the subject of debate. An alkyl branch is not a beneficial leaving group and probably requires additional steps to be removed. Their presence may suppress proper orientation and accessibility of the PAHs into dioxygenases. Generally, methyl-ethyl-naphthalenes and phenanthrenes are prevalent contaminants in the environment and, however, limited amount of studies have been done in relation to bacterial degradation. Catabolism of alkyl PAHs in aerobic bacteria suggests a very diversity of enzymes involved. These include oxidation of methyl group to alcohol, aldehyde, carboxylic acid, decarboxylation, demethylation, and dioxygenation. Even so, production of alkyl salicylate or alkylphthalate suggests that the reaction may prefer non-substituted PAH systems. However, PAHs and their alkyl derivatives can be transformed by various anaerobes through novel catabolic pathways. Proteomics have been recently employed in studies of environmental microbiology and have shown their high impact on the field of biodegradation and bioremediation.

Most studies on the microbial metabolism of PAHs have been performed with strains that use the compound under study as a growth substrate (Cerniglia, 1992; Kanaly and Harayama, 2000). However, a number of degrading bacteria act on a variety of compounds that do not

support their growth and produce partially oxidized products (Grifoll et al., 1995). This versatility is partly due to the broad substrate specificity of the degradative enzymes, as has been widely established for naphthalene and toluene dioxygenases (Wackett et al., 1988; Gibson et al., 1995). The principal objective of present work is localization of dioxygenase gene cluster, for the enzyme involved by potential marine bacterial isolates for degradation of PAHs.

2. REVIEW OF LITERATURE

Biodegradation or biotic degradation is the chemical dissolution of materials by bacteria or other biological means. The condition is often used in relation to ecology, waste material, biomedicine, and the natural environment (bioremediation) and is now commonly associated with environmentally friendly products that are capable of decomposing back into natural elements. Organic substantial can be degraded aerobically with oxygen, or anaerobically, without oxygen. A scientific term related to biodegradation is biomineralisation, in which organic matter is exchanged into minerals. Biosurfactant, an extracellular surfactant secreted by microorganisms, raises the biodegradation process.

Biodegradable matter is generally organic material such as plant and animal matter and other substances originating from living organisms, or materials that are similar enough to plant and animal matter to be use by microorganisms. Several microorganisms have a natural occurring, microbial catabolic activity to degrade, translate or accumulate a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances and metals. Major methodological in microbial biodegradation have enabled to elaborate genomic, metagenomic, proteomic, bioinformatic and other high-throughput analyses of environmentally relevant microorganisms providing unexampled insights into key biodegradative pathways and the ability of microorganisms to adapt to changing environmental conditions.

2.1. PAHs biodegradation:

Aromatic compounds can be defined as organic molecules containing 1 or more aromatic rings, generally benzene rings, for example. Various aromatic compounds co-exist as complex mixtures in petroleum refinery and distillation sites. There are three major forms: polycyclic aromatic hydrocarbons (PAHs), heterocyclic, and substituted aromatics. PAHs are a group of chemicals which contain 2 or more fused aromatic rings in linear, angular, or cluster pattern (Cerniglia, 1992; Cheung et al., 2001). Physical and chemical properties of PAHs vary with the number of rings and molecular weight. Chemical reactivity, aqueous solubility and volatility of PAHs decrease with increasing molecular weight. As a result, PAHs vary in their transport, arrangement and fate in the environment and their effects on biological group. The US EPA has identified 16 PAHs as main pollutants. Some PAHs are considered to be possible or probable

human carcinogens, and hence their distributions in the environment and possible exposure to humans have been of concerns (Menzie et al., 1992). High molecular-weight PAHs are paid particular attention as they are recalcitrant (Cheung et al., 2001). In general, PAHs are relatively stable and recalcitrant in soils and less easy to degrade than many other organic compounds. They are basically difficult to remove from contaminated soil using the treatments that have been used successfully to clean soils contaminated with more degradable or volatile organic compounds such as alkanes (Pitter and Chudoba, 1990). Three major sources of PAHs are petrogenic, pyrogenic and biogenic. Petrogenic PAHs are mostly found in petroleum site. Petroleum-derived products, are often marked as in abundance of alkyl substituted PAHs such as alkyl naphthalene, alkyl phenanthrene and alkyl dibenzothiophene. Pyrogenic PAHs are developed from combustion processes and are comprised of predominantly unsubstituted PAHs. Biogenic aromatic compounds comprising aromatic amino acids, lignin compounds and the derivatives are of biotransformation origin.

PAHs may accumulate in high concentrations in terrestrial environments near coal gasification sites and tar oil distillation plants (Capotori, 2004). Major sources of PAHs are incomplete combustion of organic materials, gas production, wood treatment facilities, and waste incineration (Cerniglia, 1992; Ahn et al., 1999; Kanaly and Harayama, 2000; Kim et al., 2003). PAHs are formed naturally during thermal geologic reactions associated with fossil-fuel and mineral production, and during burning of vegetation in forest and bush fires (Juhász and Naidu, 2000; Wilson and Jones, 1993). Anthropogenic sources, particularly fuel combustion, automobiles, spillage of petroleum products, and waste incinerators are significant sources of PAHs into the environment. Tobacco cigarette smoking is a significant source of PAH exposure to smokers and secondary smokers. PAHs generated during anthropogenic combustion activities are primarily transported via atmospheric deposition (Nishioka et al., 1986; Freeman and Cattell, 1990). Petroleum refining and transport activities are major contributors to localized loadings of PAHs into the environment. Such loadings may occur through discharge of industrial effluents and through accidental release of raw and refined products (Kanaly and Harayama, 2000). Alkyl PAHs (e.g., methylnaphthalene) have increasingly become an environmental concern. Because alkyl substitution causes a substantial decrease of water solubility, alkyl PAHs tend to be bioaccumulative. They are abundant in fossil fuels, crude oil and petroleum derived products. (Boylan and Tripp, 1971) identified a large number of aromatic hydrocarbons (e.g., alkyl-

benzenes and naphthalenes) in seawater extracts of several crude oils and kerosene. (Alexander et al., 1985) suggested that methylnaphthalenes may be useful indicators of thermal maturity of sedimentary organic matter. Methylnaphthalenes in a petroleum oil fraction can be used as active ingredient of repellent to control mosquitoes (Wirtz et al, 1981). Alkylbenz[a]anthracene, especially 7,12-dimethylbenz[a]anthracene, is a potent carcinogen in rodent skin and mammal cells (DiGiovanni, 1992).

2.2 Bacterial catabolism of PAHs:

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PAH degradation, exist in a single bacterium (Resnick et al., 1996; Liang, 2006). Multiple catabolic pathways can produce various metabolites with structural diversities, which probably are metabolised by different types of enzymes. Dioxygenases involving in dibenzo-*p*-dioxins degradation catalyze predominantly an angular dioxygenation to produce tri-hydroxy diphenyl ethers. PCDDs are well known pollutants with extravagant toxicity, particularly tetra to hexachloro analogues. Some bacterial species can catabolise upto hexachlorodibenzo-*p*-dioxins (Habe et al., 2002).

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2.3 Degradation pathways of PAHs:

Naphthalene

Naphthalene has often been used as a model compound to investigate the ability of bacteria to degrade PAHs because it is the simplest and the most soluble PAH (Goyle and Zylstra, 1997). Therefore, information of bacterial degradation of naphthalene has been used to predict pathways in the degradation of three or more ring PAHs. Numerous bacteria that have been isolated and utilize naphthalene as a sole source of carbon and energy belong to the genera *Alcaligenes*, *Mycobacterium*, *Polaromonas*, *Pseudomonas*, *Ralstonia*, *Rhodococcus* and *Streptomyces*.

Degradation of naphthalene starts through the multicomponent enzyme, naphthalene dioxygenase (Simon et al., 1993), attack on the aromatic ring to form *cis*-(1R, 2S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol). The *cis*-naphthalene dihydrodiol formed by naphthalene dioxygenase is subsequently dehydrogenated to 1,2-dihydroxynaphthalene by *cis*-dihydrodiol dehydrogenase (Auger et al., 1995). After that, 1,2-dihydroxynaphthalene is metabolized to salicylate via 2-hydroxy-2*H*-chromene-2-carboxylic acid, *cis*-o-hydroxybenzalpyruvate, and 2-hydroxy-benzaldehyde (Boboshin et al., 2008). Also, 1,2-dihydroxynaphthalene is nonenzymatically oxidized to 1,2-naphthaquinone. Salicylate is typically decarboxylated to catechol, which is further metabolised by ring fission in *meta*- and *ortho*-pathways. It was reported that salicylate (Fuenmayor et al., 1998) is converted to gentisate by salicylate-5-hydroxylase. Recently, (Jouanneau et al., 2007) purified the salicylate 1-hydroxylase from *Sphingomonas* sp. strain CHY-1 and characterised its biochemical and catalytic properties. The bacterial degradation of naphthalene has been well characterized for the catabolic enzyme system encoded by the plasmid NAH7 in *Pseudomonas putida* G7. NAH7 has two operons that contain the structural genes for naphthalene degradation. First operon contains the gene for the upper catabolic pathway encoding the enzymes necessary for the conversion of naphthalene to salicylate. Second operon contains the gene for the lower catabolic pathway encoding the enzymes necessary for the metabolism of salicylate through the catechol meta-cleavage pathway to pyruvate and acetaldehyde (Denome et al., 1993). The complete sequence structure of plasmid NAH7 (82,232 bp) was determined by (Saita et al., 2000). Also, 83,042 bp sequence of the circular naphthalene degradation plasmid pDTG1 from *Pseudomonas putida* NCIB 9816-4 was determined by Dennis. (Parales et al., 2003) reported that aspartate in the catalytic domain of naphthalene dioxygenase is a necessary residue in the major pathways of electron transfer to mononuclear iron at the active site.

Fluorene

Fluorene having three rings is a major constituent of fossil fuels and coal. Many bacteria able to use fluorene as their sole source of carbon and energy have been isolated and are in the genera of *Arthrobacter*, *Brevibacterium*, *Mycobacterium* and *Pseudomonas*.

1,2-dioxygenation of fluorene forms fluorene-1,2-diol which is transformed to 3-chromanone via 2-hydroxy-4-(2-oxo-indan-1-ylidene)-2-butenic acid, 1-formyl-2-indanone and 2-indanone-1-carboxylic acid. The second pathway begins at 3,4-dioxygenation of fluorene

leading to salicylate formation through 2-hydroxy-4-(1-oxo-indan-2-ylidene)-2-butenic acid, 2-formyl-1-indanone, 1-indanone-2-carboxylic acid, 1-indanone, 2-chromanone, and 3-(2-hydroxyphenyl)-propionic acid. The third pathway starts from C-9 monooxygenation in *Brevibacterium* sp. and *Pseudomonas* sp. This pathway is only productive if a subsequent angular carbon dioxygenation take place, leading to the formation of phthalate that is transformed to protocatechuate. Several genes involved in the degradation of fluorene to phthalate were characterized in *Terrabacter* sp. by (Habe et al., 2004).

Phenanthrene

Phenanthrene, which is a three aromatic ring system and mostly found in high concentrations in PAH contaminated sediments, surface soils, and waste sites. Bacterial degradation of phenanthrene has been studied. Numerous of bacterial strains such as *Acidovorax*, *Arthrobacter*, *Brevibacterium*, *Mycobacterium* and *Pseudomonas* have been characterised and have the ability to utilize phenanthrene as a sole carbon and energy source.

Phenanthrene contains bay- and K-regions able to form an epoxide, which is suspected to be an carcinogen. Therefore, it is used as a model substrate for studies on the catabolism of bay- and K-region containing carcinogenic PAHs such as benzo[*a*]pyrene, benzo[*a*]anthracene, and chrysene. In general, bacterial degradation of phenanthrene is initiated by 3,4-dioxygenation to give *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, which changes enzymatic dehydrogenation to 3,4-dihydroxyphenanthrene. The diol is later on catabolised to naphthalene-1,2-diol by both *ortho*-cleavage to form 2-2-carboxy-vinyl-naphthalene-1-carboxylic acid and *meta*-cleavage to form 4-1-hydroxy-naphthalen-2-yl-2-oxo-but-3-enoic acid, phthalic acid, 3,4-dihydroxyphthalic acid, protocatechuic acid and *trans*-2,3-dioxo-5-2'-hydroxyphenyl-pent-4-enoic acid. Recently, (Seo et al., 2006) elucidated that the *ortho*-cleavage part, 2-2-carboxy-vinyl-naphthalene-1-carboxylic acid, is degraded to form naphthalene-1,2-diol by naphthalene-1,2-dicarboxylic and 1-hydroxy-2-naphthoic acid. (Pagnout et al., 2007) isolated and characterised a gene cluster involved in phenanthrene degradation through 3,4-phenanthrene dioxygenation and *metac*leavage.

It is possible that 1,2-dioxygenation of phenanthrene cleave to form *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene, which undergoes enzymatic dehydrogenation to 1,2-dihydroxyphenanthrene. This diol is also subsequently catabolised to naphthalene-1,2-diol by

both *ortho* and *meta*-cleavages. Generally, phenanthrene-1,2- and 3,4-diols majorly undergo *meta*-cleavage due to the rapid accumulation of 5,6- and 7,8-benzocoumarin. Naphthalene-1,2-diol converted from 1-hydroxy-2-naphthoic acid and 2-hydroxy-1-naphthoic acid is further degraded in a phthalic acid pathway through *ortho*-cleavage and a salicylic acid pathway through *meta*-cleavage. Mallick reported that a novel *meta*-cleavage of 2-hydroxy-1-naphthoic acid to *trans*-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid in *Staphylococcus* sp. Interestingly, phenanthrene degradation starts from 9,10-dioxygenase to bear phenanthrene *cis*-9,10-dihydrodiol which is further catabolised to 2,2'-diphenic acid via 9,10-dihydroxyphenanthrene (Seo et al., 2006).

Pyrene

Pyrene possessing four benzene rings is a byproduct of gasification processes and other incomplete combustion processes. Many bacterial isolates able to degrade pyrene which have been studied. *Mycobacterium* as Gram-positive species has been most widely studied for degrading pyrene by using it as a sole carbon and energy source. *Mycobacterium* spp. are known to have high cell surface hydrophobicity and adhere to the emulsified solvent droplets. Other pyrene degrading strains isolated include *Rhodococcus* sp., *Bacillus cereus*, *Burkholderia cepacia* (Juhasz et al., 1997), *Cycloclasticus* sp. P1, *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, *Sphingomonas* sp. VKM B-2434, *Sphingomonas paucimobilis*, and *Stenotrophomonas maltophilia*. (Heitkamp et al., 1988) found the 3 products of ring oxidation, pyrene-*cis*-4,5-dihydrodiol, pyrene-*trans*-4,5-dihydrodiol and pyrenol. And 4 products of ring fission, 4-hydroxyperinaphthenone, 4-phenanthroic acid, phthalic acid, and cinnamic acid by numerous analyses, including UV, infrared, mass spectrometry, NMR, and Gas Chromatography. The formation of pyrene-*cis*-4,5-dihydrodiol by dioxygenase and pyrene-*trans*-4,5-dihydrodiol by monooxygenase proposed multiple initial oxidative attacks on pyrene. Pyrene-1,2-diol derive from the dioxygenation at pyrene 1,2-C positions is metabolised to 4-hydroxyperinaphthenone via *cis*-2-hydroxy-3-(perinaphthenone-9-yl)-propenic acid and 2-hydroxy-2*H*-1-oxa-pyrene-2-carboxylic acid. (Kim and Freeman, 2005) found 1,2-dimethoxypyrene as a product of pyrene-1,2-diol. Pyrene-4,5-diol is degraded by *orthocleavage* to phenanthrene-4,5-dicarboxylic acid, which is metabolised through phenanthrene-3,4-diol and 6,6'-dihydroxy-2,2'-biphenyl-dicarboxylic acid pathway. *Meta*-cleavage part of pyrene-4,5-diol lead to 5-hydroxy-5*H*-4-oxa-pyrene-5-

carboxylic acid *via* 2-hydroxy-2- (phenanthrene-5-one-4-enyl)-acetic acid. A novel metabolite, 6,6'-dihydroxy-2,2'- biphenyl-dicarboxylic acid, was named by (Vila et al., 2001) from the degradation of pyrene by *Mycobacterium* sp. strain AP1. (Liang et al., 2006) described pyrene-4,5-dione formation and identified almost all the enzymes required during the initial steps of pyrene degradation in *Mycobacterium* sp. (Kim et al., 2007) identified 27 enzymes necessary for constructing a complete pathway for pyrene degradation from both genomic and proteomic data. The present review reveals that the PAHs biodegradation was positively correlated to the level of PAH contamination in water and sediment samples.

3.OBJECTIVE

1. Isolation and screening for Pyrene and Anthracene degrading Bacteria.
2. To study the degradation of Pyrene and Anthracene by isolates.
3. Amplification of dioxygenase gene locus.
4. Characterization of the isolates.

4. MATERIALS AND METHODS

4.1. ISOLATION :

Water sample was collected from Paradeep (N 20° 17.542' & E 86° 42.996'), Odisha in sterilized falcon tubes, kept in ice and transferred to the laboratory immediately. 1 ml of sample was suspended into Basal Minimal media (BMM) broth (Dipotassium phosphate – 7g/L, Monopotassium phosphate-2g/L, Sodium citrate – 0.50g/L, Magnesium sulphate-0.10g/L, Ammonium sulphate -1g/L) (Sambrook et al.,1989) supplemented with 100 mg/L of Pyrene and Anthracene, each as the sole carbon and energy source for growth and then it was kept at 37°C /160 rpm for 15 days. After 15 days of incubation, the inoculum was diluted and spread on Sea Water Nutrient Agar plates. The isolated colonies were selected for further screening.

4.2. SCREENING :

The BMM agar plates were prepared and each plates were incorporated with Pyrene and Anthracene. The stock solution of Pyrene and Anthracene (5mg/ml) was prepared in Hexane and it was spread on prepared BMM agar plates and the plates were kept in laminar for few minutes to dry, so that hexane will evaporate. After that, the isolated colonies were streaked over the mat of Pyrene and Anthracene. The same colonies were also inoculated in Minimal broth with 100mg/L and kept in the incubator shaker for 7 days, in order to monitor the growth and disappearance of PAH visually, which is further kept for degradation studies.

4.3. CARBON SOURCE UTILIZATION:

The strains were inoculated in freshly prepared Luria broth and incubated at 37°C for 24 hours. After incubation, the cell mass was collected and centrifuged at 4 °C/7000 rpm for 10 minutes. Then supernatant was discarded and pellet was resuspended in 1ml of BBM. 100µl of suspended cell was transferred to 5ml BMM tubes with 100 mg/l of Pyrene and Anthracene separately as a sole carbon source. 1% glucose in BMM without any PAHs was kept as control for growth. After

that, these tubes were kept at 37°C/180 rpm for 7 days. In order to monitor the growth, OD₅₉₅ were taken at regular time interval i.e 0 day, 1 day, 3 day, 5 day & 7 day.

4.4. STANDARD CURVE OF PYRENE AND ANTHRACENE :

Stock solution of Pyrene and Anthracene was prepared in hexane at concentration of 5mg/ml. From the stock solution, 5ml of pyrene and anthracene working solution in hexane with concentration of 0.5µg/ml, 1µg/ml, 10µg/ml, 50µg/ml and 100µg /ml was prepared in triplicated for standard curve. It was scan to get the λ_{\max} value and absorbance in between 200-400nm using UV-Visible Spectrophotometer.

4.5. DEGRADATION STUDY:

Degradation was carried out by inoculating strains into test tubes containing 5 ml of Minimal Medium broth which is supplemented with PAH (Pyrene and Anthracene) at concentration of 100mg/L (Kiyohara et al, 1982). PAH was delivered to media from stock solution (5mg/ml) in hexane. It was kept for few hours to completely remove hexane from the media. Thereafter BMM-PAH tubes were subsequently incubated at 37°C/180rpm for 7 days .After 7 days, cell were harvested by centrifugation and it was again transferred to BMM-PAH tubes (Pyrene and Anthracene) at concentration of 200mg/L .Thereafter these tubes were incubated at 37°C/180 rpm for another 7 days.

After 7 days, BMM-PAH tubes were centrifuged for 10 min at 6000 rpm. The supernatant was discarded and the pellet was washed with saline water for 2 times. To above pellet autoclaved basal minimal media was added without any carbon source. Then the OD was taken at 595 nm and adjusted to 0.1. Form 0.1 OD adjusted culture 500µl was transferred to BMM media with 100mg/l of PAH and incubated at 37°C/ 160 rpm for 7 days. After 7 days the whole media was used for extraction and quantification of residual PAH (Pyrene and Anthracene)

4.6. EXTRACTION AND QUANTIFICATION:

Residual PAH was extracted from culture (5 ml) with equal volume of Dichloro methane (DCM). For extraction equal volume of DCM was added to the degradation setup. The tubes were then vortexed for 10 min and kept for another 10 min to separate aqueous and organic phase. Upper organic layer was extracted and dried over sodium sulfate. There after organic phase was pipette out and kept for drying over night. The residual was resuspended in equal volume of n-hexane. The extracted Pyrene and Anthracene was 10 times diluted in n-hexane. The residual Pyrene and Anthracene concentration was determined from standard curve of respective PAH. Absorbance of Pyrene and Anthracene was taken at 335nm and 254nm respectively

4.7. CHARACTERIZATION OF THE ISOLATES:

4.7.1. GRAM STAINING:

Gram reaction :

A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air-dried and then heat fixed. Then it was subjected to the following staining reagents:

- Flooded with Crystal violet for 1 min. followed by washing with running distilled water.
- Again, flooded with Gram's Iodine for 1 min. and washed with running distilled water.
- Then the slide was flooded with Gram's decolourizer for 30 seconds.
- After that the slide was counter stain with Safranin for 30 seconds, and washed with running distilled water.
- The slide was air dried and cell morphology was checked under microscope.

4.7.2. BIOCHEMICAL TESTS:

Sugar fermentation tests:

The sugar test was done by using Bacillus Hi-media biochemical kit (A, B1 and C) which contain different sugars. Kit A consists of Dextrose, Lactose, Maltose, Fructose, Raffinose, Xylose, Galactose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose. Kit B consists of Inulin, Sodium gluconate, Dulcitol, Glycerol, Inositol, Salicin, Sorbitol, Mannitol, Adonitol,

Arabitol, Erythritol, α -methyl-D-glucoside. Kit C consists of Rhamnose, Melezitose, α -methyl-D-mannoside, Cellobiose, Xylitol, ONPG, Esculin hydrolysis, D-Arabinose, Citrate utilization, Malonate utilization, Sorbose. Sugar fermentation test was basically used to detect bacteria's ability to ferment sugar and produce gas and/or acid end product.

Swimming and Swarming motility tests:

Swimming and swarming motility is a rapid and a coordinated translocation of a bacterial population across solid or semi-solid surfaces. This sort of motility is an example of an emerging concept in microbiology: bacterial multicellularity. Swarming motility was first reviewed by (Henrichsen, 1972) and has been mostly studied in genus *Serratia* (Alberti and Harshey, 1990), *Salmonella* (Harshey, 1994), *Aeromonas* (Kirov et al., 2002), *Bacillus* (Kearns and Losick, 2004), *Yersinia* (Young et al., 1999), *Pseudomonas* (Daziel et al., 2003, Trembley et al., 2007), *Proteus* (Caiazza et al., 2005), *Vibrio* (Rather, 2005) and *Escherichia* (Harshey and Matsuyama, 1994). This multicellular behavior has been mostly observed in controlled laboratory conditions and relies on two critical elements: 1) the nutrient composition and 2) viscosity of culture medium (i.e. % agar). One particular feature of this type of motility is the formation of dendritic fractal-like patterns formed by migrating swarms moving away from an initial location.

For swimming motility test 0.2% of nutrient agar was prepared and for swarming motility test 0.5% of nutrient agar was prepared, after that both were poured into plates. The poured plates were in semi-solid condition and 10 μ l of culture was inoculated at the centre of the plates, then the plates were kept in the incubator for 24 hrs at 37°C.

4.7.3. ANTIBIOGRAM:

All the strains were tested for antimicrobial resistance by the method of Bauer et al., 1966 with antibiotic impregnated discs (Hi-Media). The MHA plates were prepared, to which isolated culture was swabbed. After that the antibiotic disc was kept over it. The following antibiotic discs with concentration of the drug as stated in the parenthesis were used, Gen: 10 μ g, AM: 30 μ g, AZM: 30 μ g, C: 50 μ g, E: 15 μ g, VA: 30 μ g, NX: 10 μ g, S: 10 μ g, MET: 5 μ g, T: 30 μ g, K: 30 μ g,

AC:10µg. After that the strains were characterized as susceptible or resistant based on the diameter of the inhibition zones around the disc.

4.8. PREPARATION OF LYSATES:

- Fresh cultures were transferred to 2 ml of previously sterilized LB and incubated at 37°C for overnight.
- 1 ml of the suspension was taken in a 1.5ml of micro centrifuge tube (MCT) and centrifuged at 7500 rpm for 10 min at 4°C.
- Supernatant was discarded and the rest of culture was added to the pellets in MCT and again centrifuged at 7500 rpm for 10 min at 4°C.
- Supernatant was discarded and 200µl of autoclaved MQ water was added and properly mixed by vortexing.
- This mixture was then centrifuged at 7500 rpm for 10 min at 4°C and the supernatant was discarded, then 200 µl of autoclaved MQ water was added to the pellets, and again resuspended by vortexing.
- The MCT was kept in water bath at 100°C for 10 min, after that MCT was kept on ice for 5 min.
- Then centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant were transferred to a new fresh MCT.
- It was then stored at -20°C and used as template in polymerase chain reaction(PCR).

4.9. AMPLIFICATION OF PAHS DIOXYGENASE LOCUS :

PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. This technique means that the sample should not be contaminated with any other DNA or previously amplified products (amplicons) that may reside in the laboratory environment.

4.9.1.Guidance in Avoiding Contamination

- DNA sample preparation, reaction mixture assemblage and the PCR process, in addition to the following reaction product analysis, should be performed in separate areas.
- A Laminar Flow Cabinet prepared with a UV lamp is recommended for preparing the reaction mixture.
- Clean gloves should be properly worn for each PCR step.
- The use of vessels and positive displacement pipettes or tips with aerosol filters for both DNA sample and reaction mixture preparation, is strongly suggested.
- The reagents for PCR should be prepared separately and used solely for this reason. Autoclaving of all solutions, except dNTPs, primers and *Taq* DNA Polymerase is suggested. Solutions should be poured in small portions and stored in PCR areas. Aliquots should be stored separately from other DNA samples.
- A control reaction, template DNA, should always be kept, to confirm the absence of contamination.

4.9.2. Preparation of Reaction Mixture

To perform several reactions, it is suggested that the preparation of a master mix containing water, dNTPs, buffer, primers and *Taq* DNA Polymerase in a single tube, which can then be aliquoted into each individual tubes. $MgCl_2$ and template DNA solutions was then added. This method of setting reactions minimize the prospect of pipetting errors and saves time by reducing the number of reagent transfer.

REAGENTS:

In order to verify the heterogeneity, the genes of the isolates were amplified.

1. Template DNA :

After quantification, DNA sample was dissolved in sterilized distilled water to get the final concentration of 100mg/μl.

2. Primers :

The primers (Aurelie et al., 2008) for the amplification of the DNA were as follows

Forward primer- (GAG ATG CAT ACC ACG TKG GTT GGA)

Reverse primer - (AGC TGT TGT TCG GGA AGA YWG TGC MGT T)

3.10X assay buffer:

10X buffer contained 1.5 Mm MgCl₂, 50Mm KCL, 20 Mm Tris Cl (pH 8) and gelatine.

4.dNTP master mix:

The mixture was prepared with 2.5 Mm each of dATP, dGTP, dTTP and dCTP to make a final concentration of 100μl and stored at -20°C.

5.Taq DNA polymerase:

Taq DNA polymerase was obtained in a stored buffer containing 50% glycerol. It was available at a concentration of 3μl.

4.9.3. Methodology :

- Genomic DNA was amplified in sterile PCR tubes with reaction volume of 25μl containing the following components-
- 10X assay buffer-2.5μl, MgCl₂ – 1.5μl, dNTP – 0.5μl, Forward primer – 0.5μl , Reverse primer- 0.5μl, Taq DNA polymerase- 1μl, Template - 4μl, autoclaved MQ water was added to make up the volume to 25μl. Table.1.
- The tubes were then placed in the thermal cycler and amplification was carried out. Then the PCR products were then stored at -20°C for further use.

Table .1. PCR Conditions:

| | | TEMPERATURE | TIME |
|-----------|----------------------|-------------|------------|
| | Initial denaturation | 95°C | 5 minutes |
| 30 CYCLES | Denaturation | 95°C | 30 seconds |
| | Annealing | 57°C | 30 seconds |
| | Extension | 72°C | 30 seconds |
| | Final extension | 72°C | 7 minute |

5. RESULT

5.1 ISOLATION AND SCREENING :

The water sample was transferred to Basal Minimal media (BMM) broth (Sambrook et al.,1989) supplement with 100 mg/L of Pyrene and Anthracene each as the sole carbon and energy source for growth which was kept in the incubator shaker at 37°C /180 rpm for 15 days. After 15 days, it was serially diluted and 100µl was spread on Sea Water Nutrient Agar plates (Table 2). After 24hrs of incubation 21 isolated colonies were observed with different colony morphology. These isolates were further screened for their potential to degrade PAHs. Preliminary screening was simply done by continuously monitoring the growth of strain on, BMM-PAH agar plates (Table 3) and BMM-PAH broth (Fig.1) for 7 days. After 7 days, among 21 isolates continuous growth was observed in 5 isolates (MP-1, MP-4, MP-9, MP-14, MP-18) and OD was found to be more than 0.5 at 595 nm.

Table 2. Growth of isolates on Sea water nutrient agar plates.

| Isolates | Growth on Sea Water Nutrient Agar Plates |
|----------|---|
| MP-1 | +++ |
| MP-2 | + |
| MP-3 | ± |
| MP-4 | +++ |
| MP-5 | — |
| MP-6 | ++ |
| MP-7 | — |

| | |
|-------|-----|
| MP-8 | ± |
| MP-9 | +++ |
| MP-10 | ++ |
| MP-11 | + |
| MP-12 | - |
| MP-13 | - |
| MP-14 | +++ |
| MP-15 | ++ |
| MP-16 | - |
| MP-17 | + |
| MP-18 | +++ |
| MP-19 | + |
| MP-20 | ++ |
| MP-21 | ± |

+++ : Excellent, ++ : Good, + : Satisfactory, ± : Variable, - : Negative

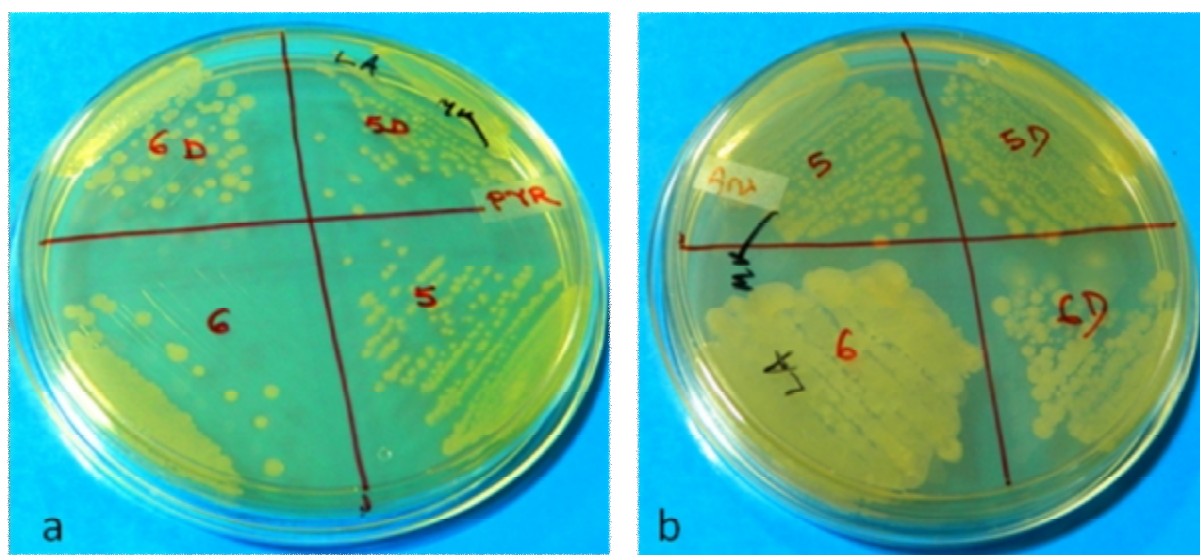


Fig.1. Isolated Colonies on basal minimal media (a) Pyrene and (b) Anthracene

Table 3. Growth of isolates on BMM-PAH agar plates.

| Isolates | Pyrene | Anthracene |
|----------|--------|------------|
| MP-1 | + | + |
| MP-4 | + | + |
| MP-9 | + | + |
| MP-14 | + | + |
| MP-18 | + | + |

5.2 CARBON SOURCE UTILIZATION:

To check the carbon source utilization, the isolates were inoculated in freshly prepared Luria broth and incubated at 37°C for 24 hours. After incubation, the cell mass was collected by centrifugation at 4 °C/7000 rpm for 10 minutes. Then supernatant was discarded and pellet was resuspended in 1ml of BBM. 100µl of suspended cell was transferred to 5ml BMM tubes with 100 mg/l of Pyrene and Anthracene separately as a sole carbon source. 1% glucose in BMM without any PAHs was kept as control for growth. After that, these tubes were kept at 37°C/160 rpm for 7 days. In order to monitor the growth, OD₅₉₅ (Fig.3) were taken at regular time interval i.e 0 day, 1 day, 3 day, 5 day & 7 day (Fig.4) by ELISA plate reader (Victor Plus)(Fig.2.). The growth pattern of isolates in term of OD₅₉₅ in different carbon source is illustrate in Table 4 and Table 5.



Fig.2. 96 well plate used to monitor the growth.

Table 4. OD₅₉₅ for Pyrene.

| Isolates | 0 DAY | 1 DAY | 3 Days | 5 Days | 7 Days |
|----------|-------|-------|--------|--------|--------|
| | | | | | |

| | | | | | |
|-------|-------|-------|-------|-------|-------|
| MP-1 | 0.011 | 0.104 | 0.229 | 0.339 | 0.5 |
| MP-4 | 0.02 | 0.2 | 0.389 | 0.415 | 0.499 |
| MP-9 | 0.022 | 0.197 | 0.303 | 0.415 | 0.495 |
| MP-14 | 0.01 | 0.221 | 0.287 | 0.346 | 0.4 |
| MP-18 | 0.012 | 0.2 | 0.299 | 0.395 | 0.487 |

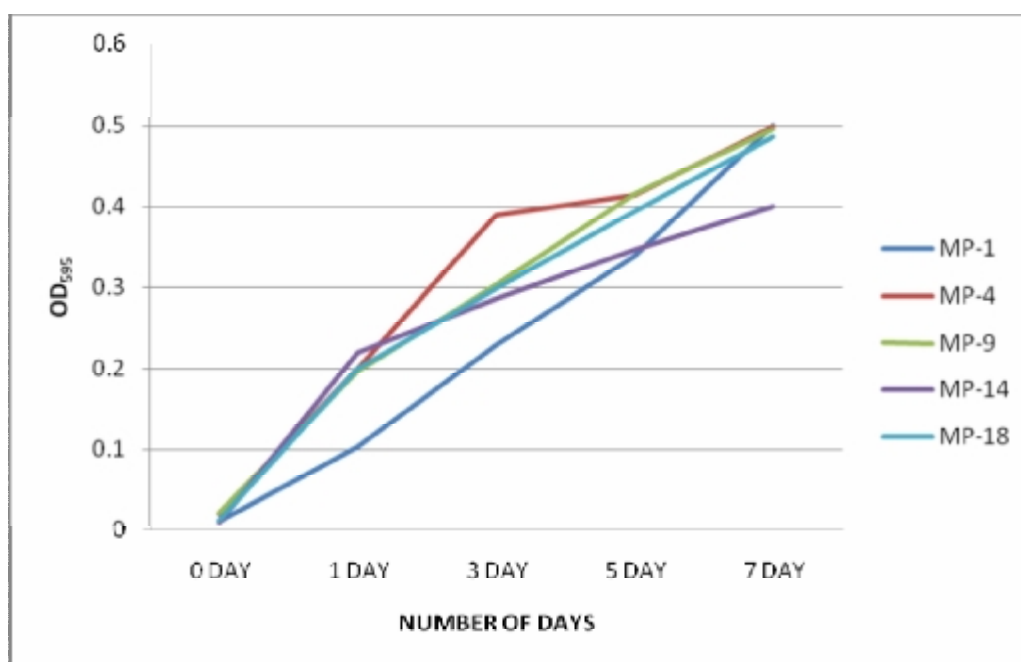


Fig.3. OD₅₉₅ on each days interval (Pyrene)

Table 5 .OD₅₉₅ for Anthracene.

| Isolates | 0 DAY | 1 DAY | 3 Days | 5 Days | 7 Days |
|----------|-------|-------|--------|--------|--------|
| MP-1 | 0.03 | 0.24 | 0.47 | 0.64 | 0.82 |
| MP-4 | 0.024 | 0.2 | 0.46 | 0.58 | 0.67 |
| MP-9 | 0.021 | 0.34 | 0.5 | 0.66 | 0.84 |
| MP-14 | 0.03 | 0.2 | 0.53 | 0.66 | 0.86 |

| | | | | | |
|-------|------|------|------|------|------|
| MP-18 | 0.03 | 0.29 | 0.46 | 0.65 | 0.79 |
|-------|------|------|------|------|------|

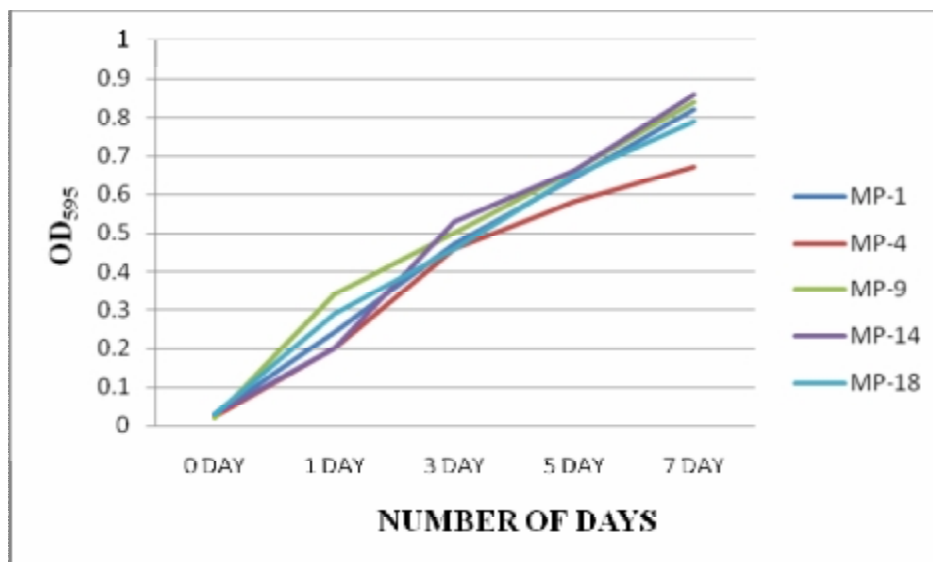


Fig.4. OD₅₉₅ on each days interval (Anthracene)

5.3. STANDARD CURVE OF PYRENE AND ANTHRACENE :

Stock solution of Pyrene and Anthracene was prepared in hexane at concentration of 5mg/ml. From the stock solution, 5ml of pyrene and anthracene working solution in hexane with concentration of 0.5µg/ml, 1µg/ml, 10µg/ml, 50µg/ml and 100µg /ml was prepared in triplicated for standard curve. Then, it was scanned to get the λ_{max} value and absorbance in between 200-400nm using UV-Visible Spectrophotometer. For Pyrene and Anthracene λ was found to be 335nm and 254nm respectively and absorbance at this λ was considered to prepare the standard curve.. λ_{max} and standard curve of Pyrene and Anthracene is shown in Fig.5 and 6. Absorbance value for different concentration is illustrated in Table 6.

Table 6. Standard Curve of Pyrene and Anthracene at different Concentration.

| CONCENTRATION | PYRENE(A ₃₃₅) | ANTHRACENE(A ₂₅₄) |
|---------------|---------------------------|-------------------------------|
| 0.5 µg | 0.0001 | 0.0001 |
| 1 µg | 0.0004 | 0.0002 |

| | | |
|---------------|-------|--------|
| 10 µg | 0.064 | 0.0741 |
| 50 µg | 0.306 | 0.323 |
| 100 µg | 0.586 | 0.592 |

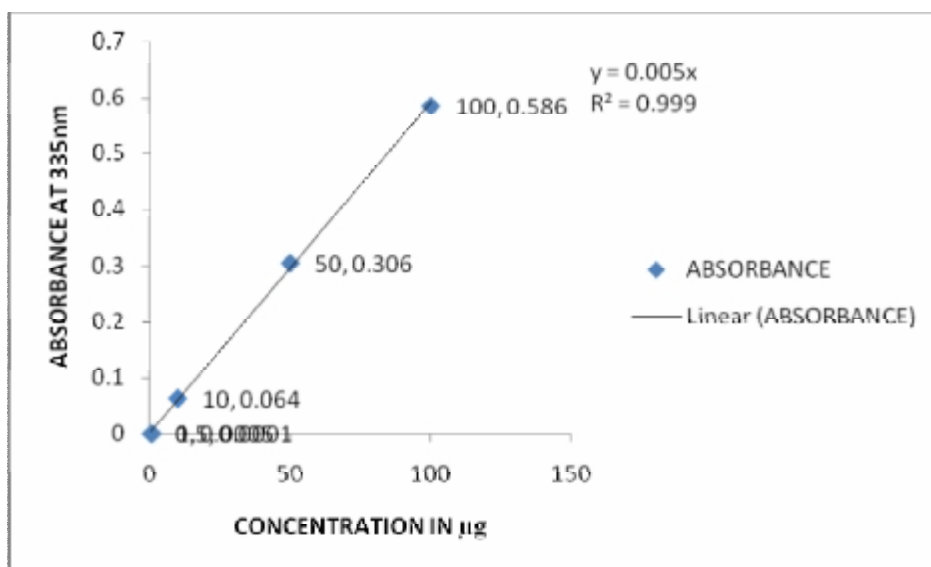


Fig .5. Standard Curve of Pyrene at different concentration.

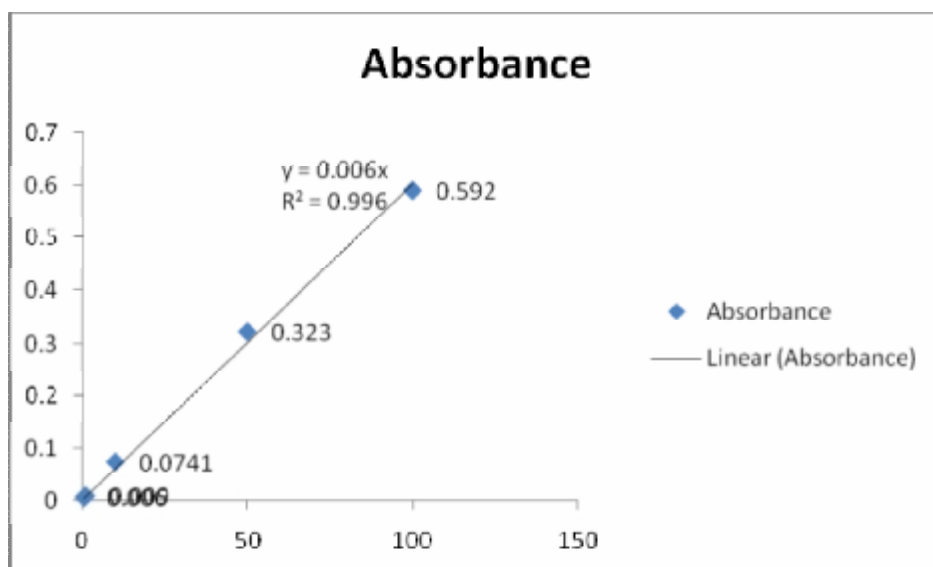


Fig .6. Standard Curve of Anthracene at different concentration.

5.4 DEGRADATION STUDY:

Degradation was carried out by inoculating strains into test tubes containing 5 ml of Minimal Medium broth which is supplemented with PAH (Pyrene and Anthracene) at concentration of 100mg/L (Kiyohara et al, 1982). PAH was delivered to media from stock solution (5mg/ml) in hexane. It was kept for few hours to completely remove hexane from the media. After that, the BMM-PAH tubes were subsequently incubated at 37°C/160rpm for 7 days. After 7 days, cell were harvested by centrifugation and it was again transferred to BMM-PAH tubes (Pyrene and Anthracene) at concentration of 200mg/L .Thereafter these tubes were incubated at 37°C/180 rpm for another 7 days.

After 7 days, BMM-PAH tubes were centrifuged for 10 min at 6000 rpm. Then,the supernatant was removed and the pellet was washed with saline water for 2 times. To above pellet autoclaved basal minimal media was added without any carbon source. Then the OD was taken at 595 nm and adjusted to 0.1. Form 0.1 OD adjusted culture 500µl was transferred to BMM media with 100mg/l of PAH and incubated at 37°C/180 rpm for 7 days. After 7 days the whole media was used for extraction and quantification of residual PAH (Pyrene and Anthracene).The results of degradation studies of these isolates was illustrated under quantification and extraction method.

5.5 EXTRACTION AND QUANTIFICATION:

Before quantification and extraction, the prepared stock at different concentration 0.5 μ g, 1 μ g, 10 μ g, 50 μ g and 100 μ g was scanned within 200 to 400 nm in UV-Visible Spectrophotometer. Then, Pyrene shows its peak at 335 nm (Fig. 7) and Anthracene show its peaks at 254 nm (Fig. 8). Then quantification and extraction was done using Dichloro-methane, then it was resuspended with hexane at 1mg/ml of concentration. After extraction each sample MP-1, MP-4, MP-9, MP-14 and MP-18, OD was taken at 334nm for Pyrene and 252 nm for Anthracene. The results of degradation studies of these isolates in Pyrene (Table 7) and Anthracene (Table 8) was shown in (Fig. 9 and 10) .The remaining concentration of both pyrene and Anthracene was calculated from standard graph of the compound.

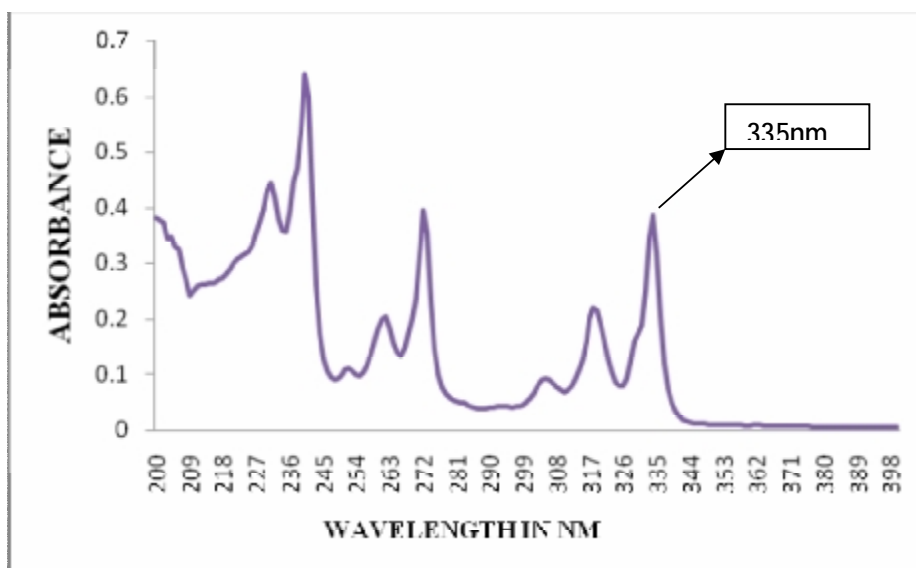


Fig.7. γ_{\max} for pyrene (335nm)

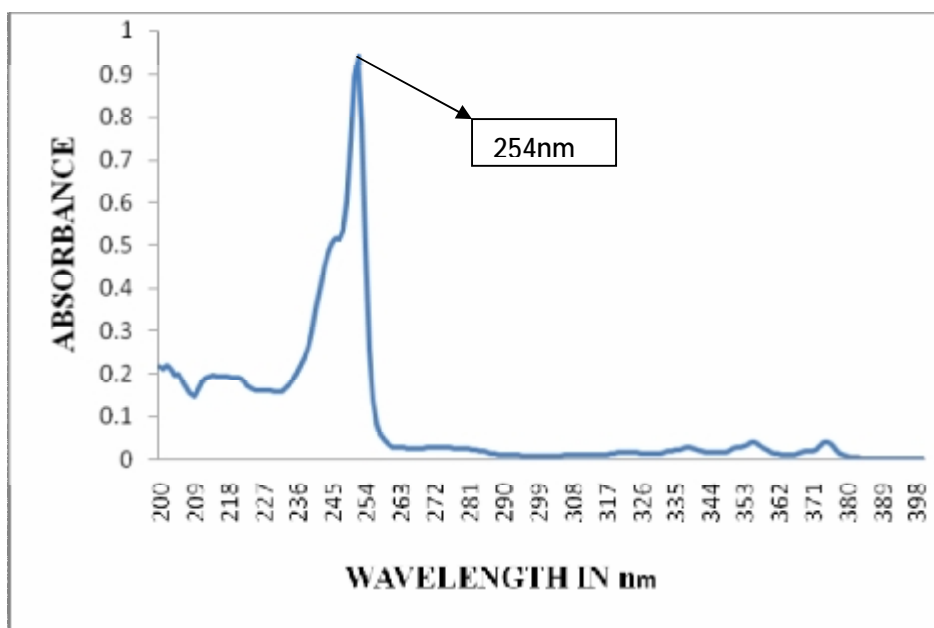


Fig.8. γ_{\max} for Anthracene (254nm)

Table 7. Percentage Degradation of Pyrene after 7 days.

| Isolates | Initial Concentration(I) mg/L | Concentration(C) mg/L after 7 days | %Degradation=$C/I \times 100$ |
|-----------------|--|---|---|
| CONTROL | 100 | 0.997 | 0.997 |
| MP-1 | 100 | 41.5366 | 58.4633 |
| MP-4 | 100 | 57.8139 | 42.1860 |
| MP-9 | 100 | 70.8106 | 29.1893 |
| MP-14 | 100 | 68.2300 | 31.7691 |
| MP-18 | 100 | 68.1499 | 31.8500 |

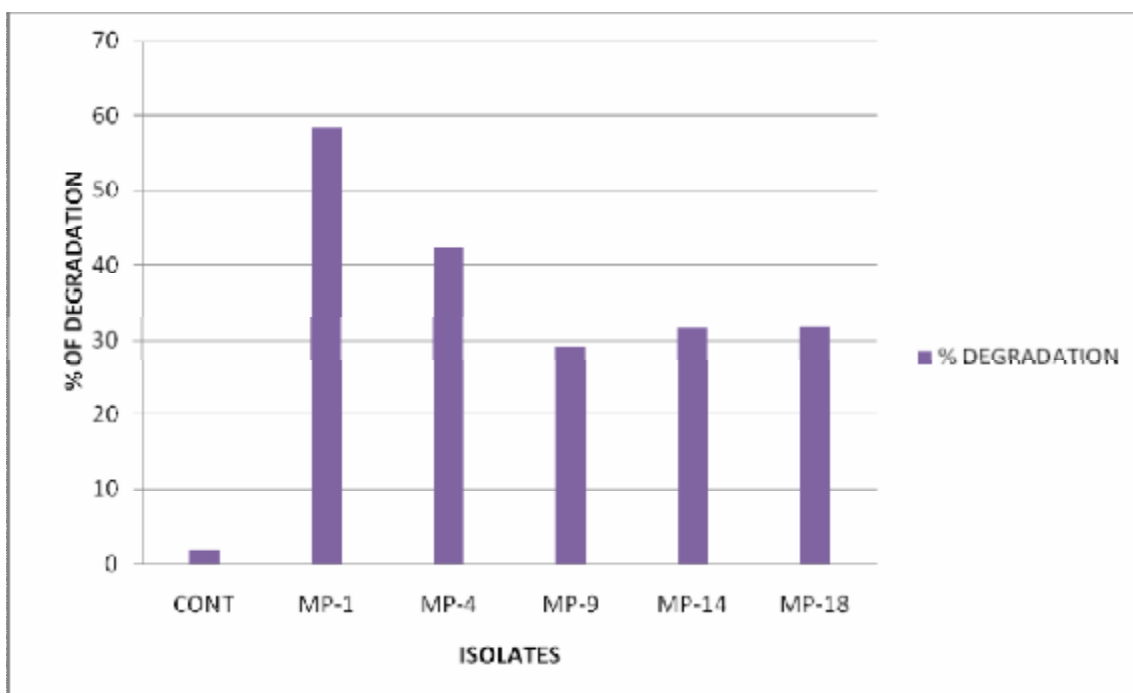


Fig .9. % Degradation of Pyrene.

Table 8. Percentage Degradation of Anthracene after 7 days.

| Isolates | Initial Concentration(I) mg/L | Concentration(C) mg/L after 7 days | %Degradation= $C/I \times 100$ |
|----------------|-------------------------------------|---------------------------------------|--------------------------------|
| CONTROL | 100 | 0.994 | 0.994 |
| MP-1 | 100 | 43.6358 | 56.3641 |
| MP-4 | 100 | 53.9531 | 46.0468 |
| MP-9 | 100 | 55.3651 | 44.6348 |
| MP-14 | 100 | 69.5418 | 30.4581 |
| MP-18 | 100 | 51.7863 | 48.2136 |

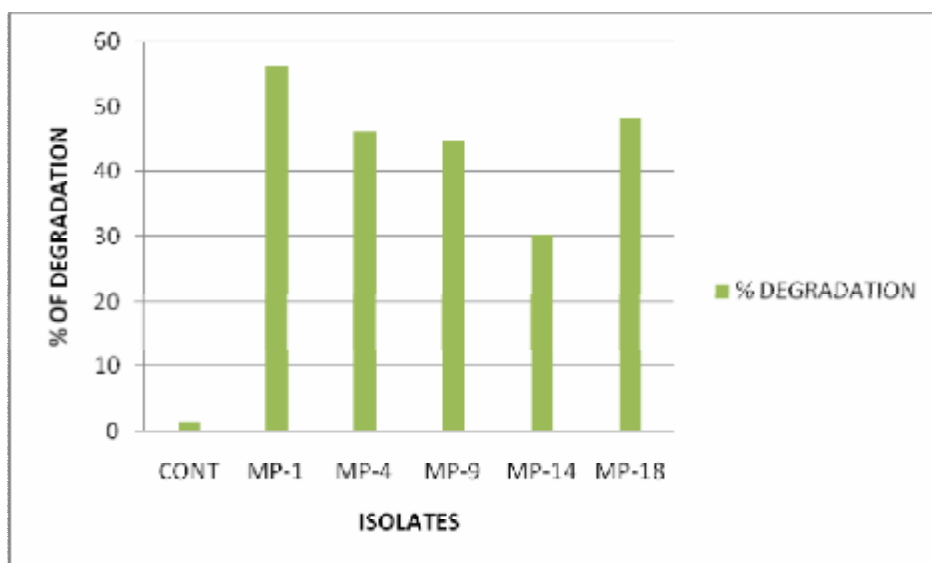


Fig .10. % Degradation of Anthracene.

5.6 CHARACTERIZATION OF ISOLATES:

All the five isolates (MP-1, MP-4, MP-9, MP-14, MP-18) were found to be Gram negative (Fig.11), Motile and show positive Citrate Utilization test. The results of characterization of these isolates was illustrated in (Table 9 and 10).

Table 9.Characterization of Isolates

| Characteristic | MP-1 | MP-4 | MP-9 | MP-14 | MP-18 |
|--------------------------|-------|-------|-------|-------|-------|
| Gram stain | - | - | - | - | - |
| Colour of colonies | Cream | White | White | Cream | White |
| Motility | + | + | + | + | + |
| Citrate utilization test | + | + | + | + | + |

+: Positive , - : Negative

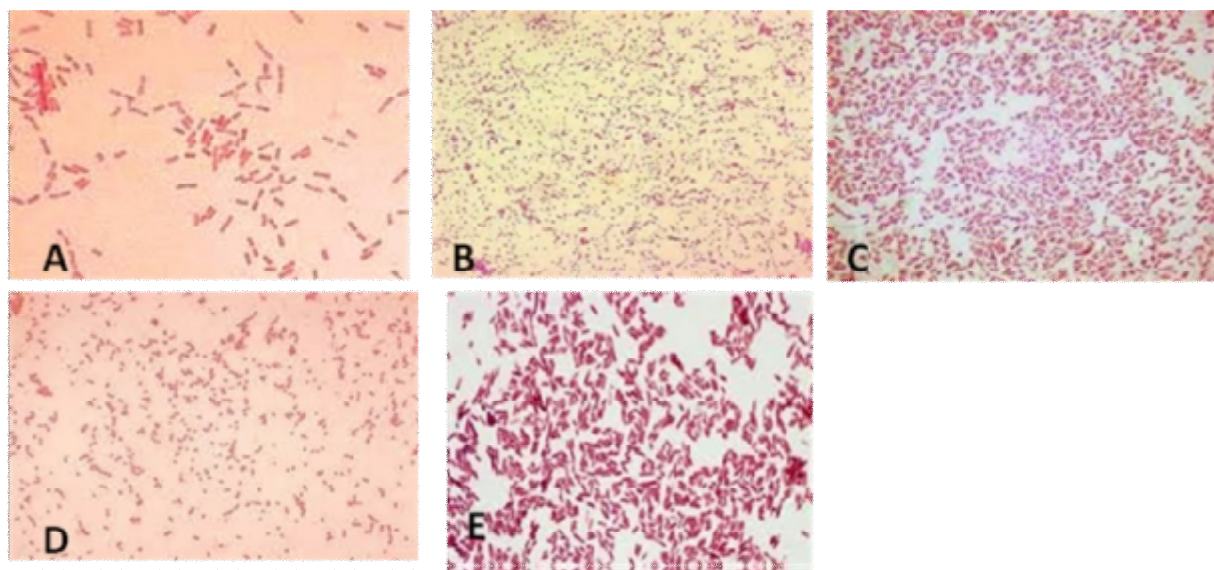


Fig. 11. Gram Staining of five Isolates. A. MP-1, B. MP-4, C. MP-9, D. MP-14, E. MP-19

Table 10. Swimming and Swarming Motility Tests:

| Motility Test | MP-1 | MP-4 | MP-9 | MP-14 | MP-18 |
|----------------|------|------|------|-------|-------|
| Swimming(0.2%) | + | + | + | + | + |
| Swarming(0.5%) | + | + | + | + | + |

+: Positive

5.6.1 CARBOHYDRATE UTILIZATION :

Sugar fermentation test was basically used to detect bacteria's ability to ferment sugar and produce gas and/or acid end product. The result of the carbohydrate utilization pattern of the five isolates was given in the (Table 11). Most of the isolates varied in the pattern of utilization of 34 different sugars.

Table 11. Sugar Fermentation Tests:

| Sugars | MP-1 | MP-4 | MP-9 | MP-14 | MP-18 |
|---------|------|------|------|-------|-------|
| Lactose | - | - | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| Xylose | - | - | - | - | - |
| Maltose | - | - | - | - | - |
| Fructose | - | - | - | - | - |
| Dextrose | - | - | - | - | - |
| Galactose | - | - | - | - | - |
| Raffinose | - | - | - | - | - |
| Trehalose | - | - | - | - | - |
| Melibiose | - | - | - | - | - |
| Sucrose | - | - | - | - | - |
| L- Arabinose | - | - | - | - | - |
| Mannose | - | - | - | - | - |
| Inulin | - | - | - | - | - |
| Glycerol | - | - | - | - | - |
| Sodium gluconate | - | + | - | - | - |
| Salicin | - | - | - | - | - |
| Dulcitol | - | - | + | - | - |
| Inositol | - | - | - | - | - |
| Sorbitol | - | - | - | - | - |
| Mannitol | - | - | - | - | - |
| Adonitol | - | + | - | - | - |
| Arabitol | - | - | - | - | - |
| Erythritol | - | - | - | - | + |
| α-Methyl-D-glucoside | - | + | - | - | - |
| Rhamnose | - | - | - | + | - |
| Cellobiose | - | - | - | - | - |
| Melezitose | - | - | + | - | - |
| α-Methyl-D-Mannoside | - | - | - | - | - |

| | | | | | |
|-----------------------------|---|---|---|---|---|
| Xylitol | - | - | - | - | - |
| ONPG | - | - | - | - | - |
| Esculin hydrolysis | + | + | + | + | + |
| D-Arabinose | - | - | - | - | - |
| Malonate utilization | + | + | + | + | + |
| Sorbose | - | - | - | - | - |

+:Positive, -: Negative

5.6.2 ANTIBIOGRAM:

All the five isolates were tested for antimicrobial resistance by the method of Bauer et al., 1966 with antibiotic impregnated discs (Hi-Media). After that, the isolates were characterized as susceptible or resistant based on the diameter (Fig .12) of the inhibition zones around the disc. Gen10, AZM 30, C 50, E 15, S 10, T 30 and K 30 were sensitive to all five isolates but AM 30 and AC 10 are resistant in MP-1 and MP-14, VA 30 is resistant in MP-1, MP-4 and MP-9, NX 10 is resistant in MP-1 and all 4 isolates were sensitive, MET 5 are resistant in all the five isolates (Table 12 and 13).

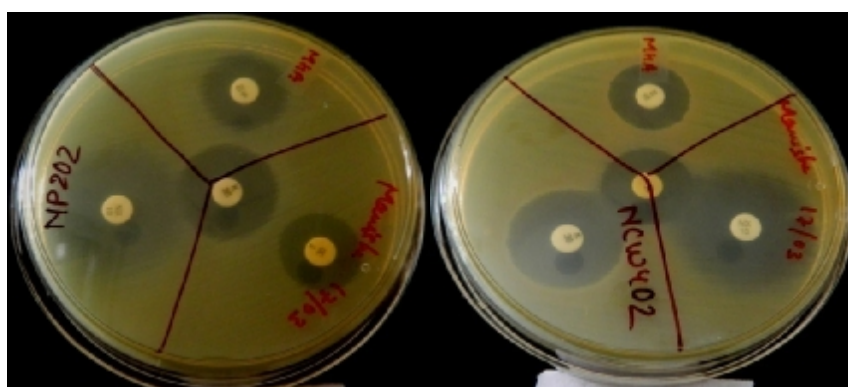


Fig.12. Inhibition of bacterial growth on MHA by antibiotic discs in disc diffusion technique.

Table 12. Zone (mm) of inhibition of antibiotics against five isolates.

| Isolates | GEN | AM | AZM | C 50 | E 15 | VA | NX | S | MET | T | K | AC |
|----------|-----|----|-----|------|------|----|----|----|-----|----|----|----|
| MP-1 | 16 | 0 | 18 | 17 | 19 | 0 | 0 | 12 | 0 | 28 | 19 | 0 |
| MP-4 | 29 | 9 | 29 | 26 | 28 | 0 | 24 | 17 | 0 | 19 | 24 | 13 |
| MP-9 | 28 | 9 | 3 | 19 | 24 | 0 | 25 | 16 | 0 | 19 | 24 | 13 |
| MP-14 | 23 | 0 | 3 | 21 | 22 | 9 | 32 | 20 | 0 | 22 | 19 | 0 |
| MP-18 | 21 | 19 | 24 | 19 | 15 | 16 | 16 | 19 | 0 | 24 | 14 | 22 |

Gen 10- Gentamicin, Amp 30- Amphotericin , Azi 30- Azithromycin, Chl 50- Chloramphenicol, Ery 15- Erythromycin, Van 30- Vancomycin, Nor 10- Norfloxacin, Str 10- Streptomycin, Met 5- Methicillin, Tet 30- Tetracyclin, Kan 30- Kanamycin, Ac 10- Ac.

Table 13. Antibiotic Resistance Pattern.

| Strain No. | Strain Name | Antibiotic Resistance Pattern |
|------------|-------------|--|
| 1 | MP-1 | AC ^R ,MET ^R ,NX ^R ,VA ^R ,AM ^R |
| 2 | MP-4 | MET ^R ,VA ^R |
| 3 | MP-9 | MET ^R ,VA ^R |
| 4 | MP-14 | AM ^R ,MET ^R ,AC ^R |
| 5 | MP-18 | MET ^R |

R: Resistant.

5.7. AMPLIFICATION OF PAHS DIOXYGENASE LOCUS :

Polymerase chain reaction (PCR) amplification with PAH-specific primers revealed the presence of a dioxygenase gene in MP-1(Fig.13).The presence of dioxygenase gene may provide opportunities to explore how bacteria develop the abilities to degrade high-molecular-weight polycyclic aromatic hydrocarbons. The size of the product was found to be 300bp.

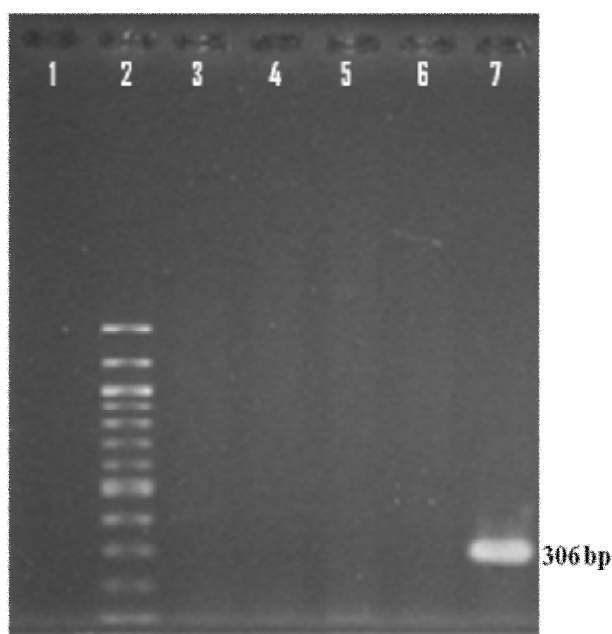


Fig.13. Gel Photograph showing amplification of *dioxygenase* gene. Lane 1: -ve Control, Lane 2: 100bp ladder, Lane 3-7: MP-4, MP-9, MP-14, MP-18 and MP-1

6. DISCUSSION

The present study focused on the isolation and screening of potential Pyrene and Anthracene degrading bacteria from the marine source. Marine water and sediments receives waste from all terrestrial, atmospheric and freshwater source and being polluted by variety of organic and inorganic pollutants. After selective enrichment of PAHs degrading bacteria, 5 isolates named as MP-1, MP-4, MP-9, MP-14, MP-19 were studied for their degradation potential. . In 7 days MP-1, MP-4, MP-9, MP-14 and MP-19, found to degrade 58.4%, 42.1%, 29.1%, 31.7%, and 31.8% of Pyrene and 56.3%, 46%, 44.6%, 30.4% and 48.2% of Anthracene respectively. Ring hydroxylating dioxygenase gene was amplified by primer- Forward primer - (GAG ATG CAT ACC ACG TGG GTT GGA) and Reverse primer - (AGC TGT TGT TCG GGA AGA YWG TGC MGT T). The results obtained with the above test demonstrated that

these particular isolates degrade more potentially Pyrene than Anthracene. A total of 21 bacterial isolates were isolated from the enriched contaminated water samples, essentially on the basis of the formation of clear zones on BMM with sprayed Pyrene and Anthracene as the sole carbon source, 5 PAH degrading bacteria isolated from water sample were analyzed phenotypically for their ability to degrade PAH and whether they contain dioxygenase locus or gene which is responsible for the degradation of PAH. The analysis was designed to screen PAH degrading isolates as potential sources for degradation of PAH i.e. Pyrene and Anthracene. All isolates were subjected to PAH spray plate method, degradation, quantification. Physical and biochemical characterization were done through Gram staining, citrate utilization test, sugar utilization test, swimming and swarming motility test, antibiotic sensitivity test. All the isolates show different morphological aspects in terms of Gram staining, color of colonies, motility and citrate utilization. Among all the isolates only MP-1 is Gram positive and rest were Gram negative. This particular isolates is highly motile. All the above isolates showing positive result in Swimming and Swarming motility test. In carbohydrate utilization test, all the isolates show negative result. All isolates were subjected to the carbon source utilization process; degradation and quantification which show different types of results i.e. all strains were differentially potentially to degrade polycyclic Aromatic hydrocarbons. Standard curve of the particular PAH i.e. Pyrene and Anthracene show maximum absorbance at 335 and 254nm respectively. Degradation and quantification study suggested strong evidence about the % of degradation of isolates in PAHs. Among all the isolates MP-1 is most potential, showing 58.4 % degradation of Pyrene and 56.3% degradation of Anthracene. In previous report, the degradation rate of Anthracene was studied in four strains (*Escherichia coli*, *Soil bacterium*, *Alcaligenes sp.* and *Thiobacter subterraneus*). the average degradation of Anthracene by these four strains was found to be 28.57%, 30.19%, 26.58% and 32.11% (Kampfer and Dott, 1988). *Pseudomonas* and *Alcaligenes sp.* are the most prevailing bacteria occurring at polluted sites due to enhanced selection by high concentration of organic xenobiotics (Abd-Elsalam et al., 2006). The result of the present study confirmed the matter that many of bacterial strains, especially Gram negative bacteria were found to degrade PAH compound at various extents (Kiyohara et al., 1982), it indicates that most efficient of the PAH degrading bacteria were belong to the genus *Pseudomonas*. Recently, (Krivobok et al., 2003) reported that the pyrene induced pdoA1 and pdoB1 genes from *Mycobacterium sp.* is highly homologous to the nidA and nidB genes. These

genes encode the terminal oxygenase component of the initial aromatic ring dioxygenase, *nidA* and *nidB*, whose products catalyse the conversion of pyrene to 4,5-dihydroxy-4,5-dihdropyrene, have been cloned and sequenced from *Mycobacterium vanbaalenii* (Khan et al., 2001). These results strongly suggest that not only dioxygenase gene but also *nidA* and *nidB* genes are widely distributed among pyrene utilizing *Mycobacteria* and play an important role in degradation of PAH. The result of antibiotic sensitivity test shows, all isolates were resistant towards the antibiotic methicillin, and all isolates show different inhibition zone towards different antibiotics. The PAH compound taken for the studies were Pyrene and Anthracene. Metabolic pathway is important for degradation study. These two compounds show different degradation pathways by forming different intermediates. Polymerase chain reaction (PCR) amplification with PAH-specific primers revealed the presence of a dioxygenase gene in MP-1. The size of the product was found to be 306bp. Previously, many specific PCR primers were designed directly on the nucleotide sequence, with either no or low degeneracy, and targeted specifically for each type of PAH-dioxygenase genes (Laurie and Lloyd, 1999; Lloyd Jones et al., 1999; Wilson et al., 1999; Ferrero et al., 2002; Widada et al., 2002; Baldwin et al., 2003; Brezna et al., 2003; Dionisi et al., 2004; Johnsen et al., 2006). Many more PAH degrading bacteria were isolated from water sediments, in order to quantify the percent of degradation in the presence of dioxygenase gene, followed by trial before use.

7. SUMMARY AND CONCLUSION

The aim of the present study was “Characterization of gene for enzyme involved in PAHs Degradation” as well as to make a bank collection of strains for further screening research. Water sample was collected from Paradeep (N 20° 17.542' & E 86° 42.996'), Odisha in sterilized falcon tubes, kept in ice and transferred to the laboratory immediately. The samples which were suspended into Basal Minimal media (BMM) broth (Sambrook et al., 1989) supplemented with 100 mg/L of Pyrene and Anthracene each as the sole carbon and energy source for growth which was then kept in the incubator shaker at 37°C /160 rpm for 15 days. After 15 days of incubation, the inoculum was diluted and spread on Sea Water Nutrient Agar plates. Therefore, after 24hrs of incubation 21 isolated colonies were observed with different colony morphology. The isolates

were further screened for their potential to degrade PAHs. After 7 days of preliminary screening, among 21 isolates continuous growth was observed in 5 isolates for further degradation and designated as MP-1, MP-4, MP-9, MP-14, MP-18 and OD was found to be more than 0.5 at 595 nm. Before quantification and extraction, the prepared stock at different concentration 0.5µg, 1µg, 10µg, 50µg and 100 µg was scanned within 200 to 400 nm in UV-Visible Spectrophotometer. Then, Pyrene show its peak at 335nm (Fig .7) and Anthracene show its peaks at 254nm(Fig .8). Then quantification and extraction was done using Dichloro-methane. After extraction each sample, OD was taken at 335nm for Pyrene and 254 nm for Anthracene. MP-1 degrade 58.4% of 100 mg/l of pyrene and 56.3% of 100mg/l of Anthracene in liquid culture within 21 days. All the isolates were found to be gram negative, motile, citrate utilization test positive, esculin hydrolysis positive, malonate utilization positive and show varied in the pattern of utilization of 34 different sugars. All the five isolates were tested for antimicrobial resistance by the method of (Bauer et al., 1966) with antibiotic impregnated discs (Hi-Media). After that, the isolates were characterized as susceptible or resistant based on the diameter of the inhibition zones around the disc. Gen10, AZM 30, C 50, E 15, S 10, T 30 and K 30 were sensitive to all five isolates but AM 30 and AC 10 are resistant in MP-1, VA 30 is resistant in MP-1, MP-4 and MP-9, MET 5 are resistant in all the five isolates. Polymerase chain reaction (PCR) amplification with PAH-specific primers revealed the presence of a dioxygenase gene in MP-1. The size of the product was found to be 306bp.

8. REFERENCES

- Abd-Elsalam, H., A. Shamseldin and E.E. Hafez, 2006. PAH degradation by two native Egyptian strains *Flavobacterium sp.* and *Pseudomonas putida*. J. Appl. Sci. Res., 11: 1092-1098.
- Ahn, Y.H.; Sanseverino, J.; Sayler, G.S.(1999) Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. *Biodegradation* , 10, 149-157.

Alberti, L; Harshey, RM (1990). "Differentiation of *Serratia marcescens* 274 into swimmer and swarmer cells". *Journal of bacteriology* 172 (8): 4322–8.

Alexander, M.(2002) *Biodegradation and Bioremediation. 2nd Ed.*; Academic Press: San Diego, USA,; pp. 325-327.

Alexander, R.; Kagi, R.I.; Rowland, S.J.; Sheppard, P.N.; Chirila, T.V.(1985) The effect of thermal maturity on distribution of dimethylnaphthalenes and trimethylnaphthalenes in some ancient sediments and petroleum. *Geochim. Cosmochim. Acta.*, 49, 385-395.

Auger, R.L.; Jacobson, A.M.; Domach, M.M.(1995) Effect of nonionic surfactant addition on bacterial metabolism of naphthalene: Assessment of toxicity and overflow metabolism potential. *J. Hazard Mater.*, 43, 263-272.

Aurélien C. , Norini, M., Beguiristain, T., Leyval, C.,(2008). Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHD α) genes from Gram positive and Gram negative bacteria in soil and sediment samples. 34, 345-360.

Baboshin, M.; Akimov, V.; Baskunov, B.; Born, T.L.; Khan, S.U.; Golovleva, L.(2008) Conversion of polycyclic aromatic hydrocarbons by *Sphingomonas* sp. VKM B-2434. *Biodegradation*, 19, 567-576.

Baldwin, B.R., Nakatsu, C.H., Nies, L., 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. *Appl. Environ. Microbiol.* 69, 3350–3358.

Batie C.J, Lahaie E, Ballou D.P.(1987) Purification and characterization of phthalate oxygenase and phthalate oxygenase reductase from *Pseudomonas cepacia*. *J Biol Chem* ;262:1510–8.

Bauer, A.W.; Kirby, W.M.; Sherris, J.C.; Turck, M.(1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45(4): 493-6.

Bosch, R., E. Garcia-Valdes, and E. R. Moore. (1999). Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. *Gene* 236:149–157.

Boylan, D.B.; Tripp, B.W.(1971) Determination of hydrocarbons in sea water extracts of crude oil and crude oil fractions. *Nature* , 230, 44-47.

Brezna, B., Khan, A.A., Cerniglia, C.E., 2003. Molecular characterization of dioxygenases from polycyclic aromatic hydrocarbon-degrading *Mycobacterium* spp. *FEMS Microbiol. Lett.* 223, 177–183.

Bumpus. J.A. (1989). Biodegradation of polycyclic aromatic hydrocarbons by *Phanerochaete chrysosporium* *Appl. Environ. Microbiol.*, 61, pp. 2631–2635.

Caiazza, N. C.; Shanks, R. M. Q.; O'Toole, G. A. (2005). "Rhamnolipids Modulate Swarming Motility Patterns of *Pseudomonas aeruginosa*". *Journal of Bacteriology* 187 (21): 7351.

Capotori, G.; Digianvincenzo, P.; Cesti, P.; Bernardi, A.; Guglielmetti, G.(2004) Pyrene and benzo[a]pyrene metabolism by an *Aspergillus terreus* strain isolated from a polycyclic aromatic hydrocarbons polluted soil. *Biodegradation* , 15, 79-85.

Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3:351–368.

Cheung, P.Y.; Kinkle, B.K.(2001) *Mycobacterium* diversity and pyrene mineralization in petroleum contaminated soils. *Appl. Environ. Microbiol.*, 67, 2222-2229.

Denome, S.A.; Stanley, D.C.; Olson, E.S.; Young, K.D.(1993) Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* Strains: Complete DNA sequence of an upper naphthalene catabolic pathway. *J. Bacteriol.*, 175, 6890-6901.

Deziel, E.; Lepine, F; Milot, S; Villemur, R (2003). "rhlA is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAAs), the precursors of rhamnolipids". *Microbiology* 149 (Pt 8): 2005.

DiGiovanni, J. Multi stage carcinogenesis in mouse skin. *Pharmacol. Ther.* 1992, 54, 63-128. Henrichsen, J (1972). "Bacterial surface translocation: a survey and a classification". *Bacteriological reviews* 36 (4): 478–503.

Dionisi, H., Chewning, C., Morgan, K., Menn, F., Easter, J., Sayler, G., 2004. Abundance of dioxygenase genes similar to *Ralstonia* sp. strain U2 nagAc is correlated with naphthalene concentrations in coal tar-contaminated freshwater sediments. *Appl. Environ. Microbiol.* 70, 3988–3995.

Eaton R.W.(2001) Plasmid-encoded phthalate catabolic pathway in *Arthrobacter keyseri* 12B. *J Bacteriol* ;183:3689–703.

Ferraro, D. J., L. Gakhar, and S. Ramaswamy. (2005). Rieske business: structure-function of Rieske non-heme oxygenases. *Biochem. Biophys. Res. Commun.* 338:175–190.

Ferrero, M., Llobet-Brossa, E., Lalucat, J., Garcia-Valdes, E., Rossello-Mora, R., Bosch, R., 2002. Coexistence of two distinct copies of naphthalene degradation genes in *Pseudomonas* strains isolated from the western Mediterranean region. *Appl. Environ. Microbiol.* 68, 957–962.

Freeman, D.J.; Cattell, D.W.S.(1990) Wood burning as a source of atmospheric polycyclic aromatic hydrocarbons. *Environ. Sci. Technol.* 24, 1581-1585.

Fuenmayor, S.L.; Wild, M.; Boyes, A.L.; Williams, P.A.(1998) A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. Strain U2. *J. Bacteriol.*, 180,2522-2530.

Fujii T, TakeoM, Maeda Y.(1997) Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology*;143:93–99.

Fukmori F, Sain CP.(1997) Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22(pTDN1). *J Bacteriol* ;79:399–408.

Gibson, D. T., and R. E. Parales.(2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr. Opin. Biotechnol.* 11:236–243.

Gibson, D. T., S. M. Resnick, K. Le, J. M. Brand, D. S. Torok, L. P. Wackett, M. J. Schoken, and B. E. Haigler. (1995). Desaturation, dioxygenation, and monooxygenation reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain 9816-4. *J. Bacteriol.* 177:2615–2621.

Goyal, A.K.; Zylstra, G.J.(1997) Genetics of naphthalene and phenanthrene degradation by *Comamonas testosteroni*. *J. Ind. Microbiol. Biotechnol.*, 19, 401-407.

Grifoll M, Selifonov S.A, Gatlin C.V, Chapman P.J.(1995). Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. *Appl Environ Microbiol* ;61:3711-/23.

Grifoll, M., Casellas, M., Bayona, J. M. & Solanas, A. M. (1992).Isolation and characterization of a fluorene-degrading bacterium:identification of ring oxidation and ring fission products. *Appl Environ Microbiol* 58, 2910–2917.

Gurbiel R.J, Batie C.J, Sivaraja M, True A.E, Fee J.A.(1989) Electron nuclear double resonance spectroscopy of N-15 enriched phthalate dioxygenase from *Pseudomonas cepacia* proves that 2 histidines are coordinated to the [2Fe-2S] Rieske-type clusters. *Biochemistry*;28:4861–71.

Habe, H. & Omori, T. (2003). Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Bioscience Biotechnology Biochem* 67, 225–243.

Haimou Zhang . Aristeidis Kallimanis .Anna I. Koukkou . Constantin Drinas(2004).Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. *Appl. Microbiol Biotechnol* (2004) 65: 124–131.

Happe B, Eltis L.D, Poth H, Hedderich R, Timmis KN.(1993) Characterization of 2,2',3'-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-*p*-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. *J Bacteriol* ;175:7313–20.

Harshey, Rasika M. (1994). "Bees aren't the only ones: swarming in Gram-negative bacteria". *Molecular Microbiology* 13 (3): 389.

Harshey, RM; Matsuyama, T (1994). "Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells". *Proceedings of the National Academy of Sciences of the United States of America* 91 (18): 8631–5.

Heitkamp, M.A.; Franklin, W.; Cerniglia, C.E.(1988) Microbial metabolism of polycyclic aromatic hydrocarbons: Isolation and characterization of a pyrene-degrading bacterium. *Appl. Environ. Microbiol.*, 54, 2549-2555.

Johnsen, A.R., De Liphay, J.R., Reichenberg, F., Sorensen, S.J., Andersen, O., Christensen, P., Binderup, M.L., Jacobsen, C., et al., 2006.

Biodegradation, bioaccessibility, and genotoxicity of diffuse Polycyclic Aromatic Hydrocarbon (PAH) pollution at a motorway site. *Environ. Sci. Technol.* 40, 3293–3298.

Jouanneau, Y.; Micoud, J.; Meyer, C. (2007) Purification and characterization of a three-component salicylate 1-hydroxylase from *Sphingomonas* sp. Strain CHY-1. *Appl. Environ. Microbiol.*, 73, 7515-7521.

Juhasz, A.L.; Britz, M.L.; Stanley, G.A. (1997) Degradation of fluoranthene, pyrene, benz[*a*]anthracene and dibenz[*a,h*]anthracene by *Burkholderia cepacia*. *J. Appl. Microbiol.* , 83, 189-198.

Juhasz, A.L.; Naidu, R. (2000) Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[*a*]pyrene. *Int. Biodeter. Biodegr.*, 45, 57-88.

Kahng H.Y. (2002) Cellular responses of *Pseudomonas* sp. KK1 to two-ring polycyclic aromatic hydrocarbon, naphthalene. *J Microbiol* ;40:38–42.

Kampfer, P. and W. Dott, 1988. Systematisierung der mikrobiologischen Untersuchungen von Boden und Wasser. In: Altlasten 2 Thome'-Kozmiensky K.J. (Ed.). EF, Berlin, pp: 649-674..

Kanally, R. A. & Harayama, S. (2000). Biodegradation of high molecular-weight PAHs by bacteria. *J Bacteriol* 182, 2059–2067.

Kearns, Daniel B.; Losick, Richard (2004). "Swarming motility in undomesticated *Bacillus subtilis*". *Molecular Microbiology* 49 (3): 581.

Khan, A.A.; Wang, R.E.; Cao, W.W. (2001). Molecular cloning, nucleotide sequence and expression of genes encoding a PAHs ring dioxygenase from *Mycobacterium* sp. *Appl. Environ. Microbiol.*, 67, 3577-3585.

Kim, S.J.; Kweon, O.; Jones, R.C.; Freeman, J.P.; Edmondson, R.D.; Cerniglia, C.E.(2007) Complete and integrated pyrene degradation pathway in *Mycobacterium vanbaalenii* PYR-1 based on systems biology. *J. Bacteriol.*, 189, 464-472.

Kim, T.J.; Lee, E.Y.; Kim, Y.J.; Cho, K.S.; Ryu, H.W.(2003) Degradation of polyaromatic hydrocarbons by *Burkholderia cepacia* 2A-12. *World J. Microbiol. Biotechnol.* , 19, 411-417.

Kim, Y.H.; Freeman, J.P.(2005) Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1. *Appl. Microbiol. Biotechnol.*, 67, 275-285

Kirov, S. M.; Tassell, B. C.; Semmler, A. B. T.; O'Donovan, L. A.; Rabaan, A. A.; Shaw, J. G. (2002)."Lateral Flagella and Swarming Motility in Aeromonas Species". *Journal of Bacteriology* 184 (2): 547.

Kiyohara H, Nago K, Yana K (1982) Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. *Appl Environ Microbiol* 43:454–457.

Klein J.(2000) *Biotechnology, 2nd Ed.;; Environmental Processes II – Soil Decontamination*. Wiley-VCH: Weinheim, volume 11b.

Krivobok, S.; Meyer, C.; Willison, J.C.(2003). Identification of pyrene induced protein in *Mycobacterium sp.* evidence for two ring hydroxylating dioxygenase. *J.Bacteriol.*,185, 3828-3841.

Laurie A.D, Lloyd-Jones G. (1999) The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J Bacteriol* ;181: 531–40.

Lloyd-Jones,G.; Laurie, A.D.; Hunter, D.W.F.; Fraser, R., 1999. Analysis of catabolic genes for naphthalene and phenanthrene degradation in contaminated New Zealand soils. *FEMS. Microbiol. Ecol.* 29, 69–79.

Liang, Y.; Gardner, D.R.; Miller, C.D.; Chen, D.; Anderson, A.J.; Weimer, B.C.; Sims, R.C.(2006).Study of biochemical pathways and enzymes involved in pyrene degradation by *Micobacterium* sp. strain KMS. *Appl. Environ. Microbiol.*, 72, 7821-7828.

Menzie, C.A.; Potochi, B.B.; Santodonato, J.(1992) Exposure to carcinogenic PAHs in the environment.*Environ. Sci. Technol.*, 26, 1278-1284.

Meyer S, Moser R, Neef A, Stahl U, Ka mpfer P.(1999) Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. *Microbiology*;145:1731-/41.

Moser R, Stahl U.(2001) Insights into the genetic diversity of initial dioxygenases from PAH-degrading bacteria. *Appl Microbiol Biotechnol* ;55:609–18.

Mueller, J. G., Chapman, P. J. & Pritchard, P. H. (1989). Creosote contaminated sites. *Environ Sci Technol* 23, 1197–1201.

Nishioka, M.: Chang, H.C.: Lee, M.L.(1986) Structural characteristics of polycyclic aromatic hydrocarbons in coal tars and combustion products. *Environ. Sci. Technol.*, 20, 1023-1027.

Nojiri H.: Sekiguchi H.: Maeda K.: Urata M.: Nakai S.I.: Yoshida T.(2001).Genetic characterization and evolutionary implication of a *car* gene cluster in the carbazole degrader *Pseudomonas* sp. strain CA10. *J Bacteriol* ; 183:3663–79.

Pagnout, C.: Frache, G.: Poupin, P.: Maunit, B.: Muller, J.F.: Ferard, J.F.(2007). Isolation and characterization of a gene cluster in PAH degradation in *Mycobacterium* sp. strain SNP11: Expression in *Mycobacterium smegmatis* mc2155. *Res. Microbiol.*, 158, 175-186.

Parales, R. E. (2003). The role of active-site residues in naphthalene dioxygenase. *J. Ind. Microbiol. Biotechnol.* 30:271–278.

Pitter, P.: Chudoba, J.(1990) *Biodegradation of Organic Substances in the Aquatic Environment*. CRC Press: Boca Raton, FL, 34:247-290.

Rather, Philip, N.(2005)."Swarm cell differentiation in *Proteus mirabilis*". *Environmental Microbiology* 7 (8): 1065.

Resnick S.M.: Lee K.: Gibson D.T.(1996). Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. *J Ind Microbiol* ; 17:438-57.

Saito A.; Iwabuchi T.; Harayama S.A. (2000). Novel phenanthrene dioxygenase from *Nocardioides* sp. strain KP7: expression in *Escherichia coli*. *J Bacteriol* ; 182:2134–41.

Sambrook, J.: Fritsch, E.F.: Maniatis, T.(1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.87, 234-256.

Seo, J. S.: Keum, Y. S.: Hu, Y.: Lee, S. E.: Li, Q. X. (2006).Phenanthrene degradation in *Arthrobacter* sp. Pl-1: initial 1,2-,3,4- and 9,10-dioxygenation, and meta- and ortho-cleavages of naphthalene-1,2-diol after its formation from naphthalene-1,2-dicarboxylic acid and hydroxyl naphthoic acids. *Chemosphere* 65, 2388–2394.

Shepherd J.M.: Lloyd-Jones G.(1998) Novel carbazole degradation genes of *Sphingomonas* CB3: sequence analysis, transcription, and molecular ecology. *Biochem Biophys Res Commun* ; 247:129–35.

Simon, M. J.: Saunders, R.: Ensley,B.D.: Suggs,S.: Harcourt, A.: Suen, W.C.: Cruden, D.L.: Gibson, D.T.: Zylstra., G.J.(1993).Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. *Gene* 127:31–37.

Singh, A.: Ward, O.P. (2004). Biodegradation and Bioremediation: Series: Soil Biology, vol. 233 Springer-Verlag, New York.

Van Hamme, J. D.: Singh, A.: Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiol Mol Biol Rev* 67, 503–549.

Vila, J.: Lopez, Z.: Sabate, J.: Minguillon, C.: Solanas, A.M.: Grifoll, M. (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. Strain Ap1: Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Appl. Environ. Microbiol.* 67, 5497-5505.

Wackett, L.P.: Kwart, L.D.: Gibson, D.T. (1988). Benzylic monooxygenation catalysed by toluene dioxygenase from *Pseudomonas putida*. *Biochemistry* 27:1360–1367.

Widada, J.: Nojiri, H.: Kasuga, K.: Yoshida, T.: Habe, H.: Omori, T. (2002). Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. *Appl. Microbiol. Biotechnol.* 58, 202–209.

Wilson, M.S.: Bakermans, C.: Madsen, E.L. (1999). In situ, real-time catabolic gene expression: extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl. Environ. Microbiol.* 65, 80–87.

Wilson, S.C.: Jones, K.C. (1993). Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. *Environ. Pollut.*, 81, 229-249.

Wirtz, R.A.: Turrentine, J.D.: Fox, R.C. (1981) Area repellents for mosquitoes (Diptera: culicidae): identification of the active ingredients in a petroleum oil fraction. *J. Med. Entomol.*, 18, 126-128.

Young, G.M.: Smith, M.J.: Minnich, S.A.: Miller, V.L. (1999). "The *Yersinia enterocolitica* Motility Master Regulatory Operon, *flhDC*, Is Required for Flagellin Production, Swimming Motility, and Swarming Motility". *Journal of bacteriology* 181 (9): 2823–33.

Yuan, S.Y.: Chang, J.S.: Yen, J.H.: Chang, B.V.(2001). Biodegradation of phenanthrene in river sediment *Chemosphere*, 43, pp. 273–278